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Photoactivatable Fluorescein Derivatives Caged with a (3-Hydroxy-2-naphthalenyl)methyl Group

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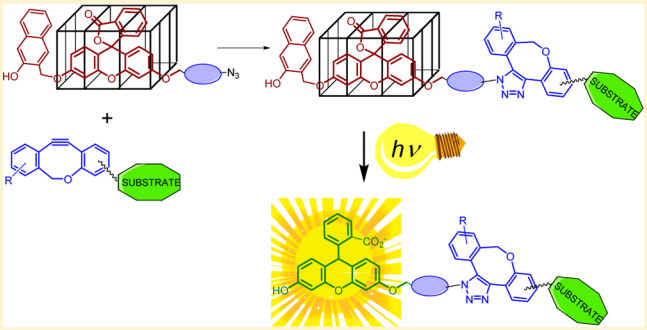
Supporting Information

ABSTRACT: The (3-hydroxy-2-naphthalenyl)methyl (NQMP) group represents an efficient photocage for fluorescein-based dyes. Thus, irradiation of the 6-NQMP ether of 2′-hydroxymethylfluorescein with low-intensity UVA light results in a 4-fold increase in emission intensity. Photoactivation of nonfluorescent NQMP-caged 3-allyloxyfluorescein produces a highly emissive fluorescein monoether. To facilitate conjugation of the caged dye to the substrate of interest via click chemistry, the allyloxy appendage was functionalized with an azide moiety.

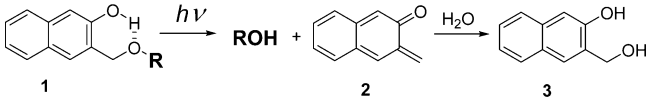


Labeling of biological substrates with fluorescent tags represents one of the most useful tools in biochemistry and cell biology.[1] Fluorogenic probes that increase the intensity (or more precisely, quantum yield) of fluorescence under the action of external stimuli add an extra dimension to these methods.[2] Thus, recently developed photoactivatable fluorophores (PAFs) permit spatiotemporal control of the emission of fluorescent reporters.[3] PAFs are crucial components of several advanced bioimaging techniques, such as local activation of fluorescent molecular probes (LAMP),[4] photoactivated localization microscopy (e.g., PALM, FPALM, STORM),[5,6] and other methods of ultraresolution microscopy.[7] PAFs have also found use in protein tracking/trafficking studies in cells[8] as well as fate mapping of cells in organisms.[9]

Several successful approaches for the design of PAFs have been reported recently. Enhancement of fluorescence efficiency can be accomplished by photochemical rearrangement of the precursor molecule that increases the π conjugation,[10] and push−pull fluorophores can be turned on by light-induced changes in the electronic properties of substituents.[11] The blinking properties of some fluorescent proteins can be employed to achieve switchable emission.[12] However, the most popular strategy in PAF design is caging, that is, the use of photolabile protecting groups (PPGs)[13] to alter the emission of conventional fluorescent dyes. Irradiation of the caged fluorophore removes the PPG and releases the dye.[6,14,15] The main advantage of the latter approach is its compatibility with well-developed fluorescence imaging techniques. While *o*-nitrobenzyl-based PPGs are the most commonly employed in the development of PAFs,[6,14] this family of PPGs has some significant drawbacks. Substrate release from the *o*-nitrobenzyl cage is often slow, as it proceeds through several dark steps,[16] and its efficiency depends on the pH of the solution and the basicity of the caged functionality.[13] In addition, uncaging is accompanied by the formation of reactive and cytotoxic *o*-nitrosobenzoyl byproducts.[13] To alleviate these complications, the utility of other caging groups is being actively explored.[15]

In this report, we describe the use of the recently developed (3-hydroxy-2-naphthalenyl)methyl (NQMP)[17] PPG for the caging of fluorescein-based dyes. Irradiation of NQMP ethers or esters **1** with 300−350 nm light results in the efficient cleavage of the benzylic C−O bond and release of the substrate (Scheme 1). The *o*-naphthoquinone methide (*o*NQM)

Scheme 1. Photochemical Substrate Release from the NQMP Cage



intermediate **2** is rapidly ($\tau \sim 7$ ms) hydrated to 3-(hydroxymethyl)naphthalen-2-ol (**3**) (Scheme 1).[18] Substrate release from the NQMP cage is efficient ($\Phi = 0.2–0.3$) and very fast ($\sim 10^5$ s^{-1}), making this PPG suitable for time-resolved experiments.

We decided to test the utility of the NQMP group for PAF development on the example of NQMP-caged fluorescein derivative **4**. We hypothesized that this compound should have low fluorescence efficiency since its vinyl ether analogue is essentially nonfluorescent at physiological pH.[19] Irradiation of **4** was expected to release highly fluorescent 6-hydroxy-9-(2-(hydroxymethyl)phenyl)-3*H*-xanthen-3-one (**5**) (Scheme 2).

Photoactivatable fluorophore **4** was synthesized in four steps from fluorescein (Scheme 3). Esterification of the latter was

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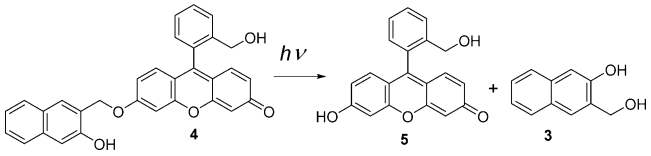
Scheme 2. Photorelease of 6-Hydroxy-9-(2-(hydroxymethyl)phenyl)-3*H*-xanthen-3-one (5) from the NQMP Cage



achieved by refluxing it in alcohol with 1−3 molar equiv of boron trifluoride etherate. This procedure cleanly afforded the corresponding fluorescein ethyl ester **6**. It is worth noting that conventional esterification of fluorescein in the presence of strong mineral acids produces significant amounts of byproducts and requires complex workup procedures.[20] Williamson esterification of fluorescein ethyl ester **6** with ethoxymethyl (EOM)-protected 3-(bromomethyl)-3-naphthol (**7**) (prepared as described in the Experimental Section) at room temperature gave mono-NQMP-caged fluorescein ethyl ester **8** in 99% isolated yield. The ethoxycarbonyl moiety of **8** was reduced to the corresponding alcohol with DIBALH. Even mild hydride sources, (e.g., $NaBH_4$) are known to reduce the *p*-quinone methide fragment of fluorescein to the corresponding phenol.[21] To reoxidize it to the quinoid form, the crude product mixture was treated with DDQ[19a] to afford **9**. Subsequent attempts to remove the EOM (as well as MOM) protecting group from **9** using strong acids (e.g., HCl, TFA, etc.) were accompanied by the significant hydrolysis of the NQMP ether. Fortunately, incubation of **9** in 95% aqueous methanol at 40 °C in the presence of Amberlyst-15 resin[22] produced NQMP-caged dye **4** in 60% yield (Scheme 3).

The UV spectrum of mono-NQMP-caged fluorescein derivative **4** in aqueous solution at pH 7.4 (PBS buffer) shows two absorption bands at 467 and 491 nm with similar intensities (log ε = 3.7) (Figure 1). The presence of these bands suggests that caged dye **4** exists in a quinoid form to some extent. This suggestion is supported by the significant fluorescence of **4** (Φ_{FL} = 0.29−0.32) with an emission maximum at 513 nm (Figure 2). The emission efficiency of **4** is similar to that of the monoanion of fluorescein.[1b]

Irradiation of a PBS solution of caged dye **4** using a 300 or 350 nm fluorescent lamp results in the efficient release of **5** (Scheme 2), as evidenced by the rise of the characteristic UV band at 497 nm (Figure 1). HRMS-ESI of the resulting photolysate confirmed the clean release of the fluorescent dye **5**

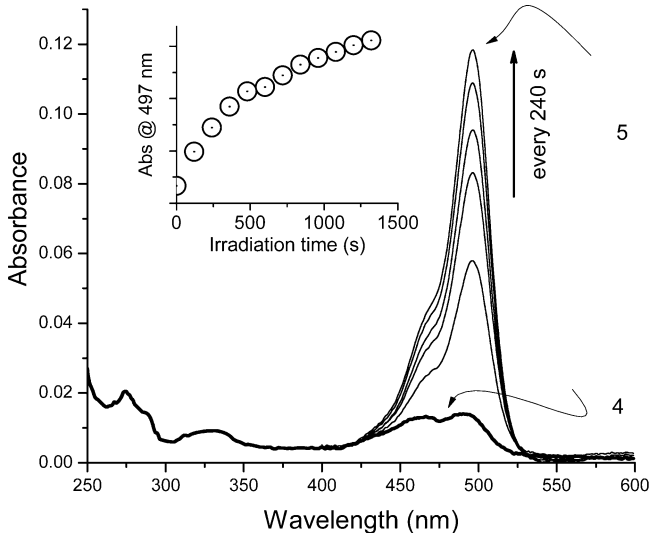


Figure 1. UV spectra of a 2.8 μM aqueous solution of **4** (in PBS buffer + 2% DMSO, pH 7.4) in the dark (thick line) and during irradiation at 350 nm (thin lines, every 240 s). The inset shows the change in absorbance at 497 nm vs irradiation time.

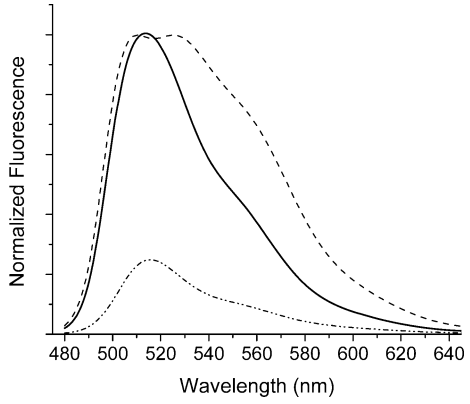
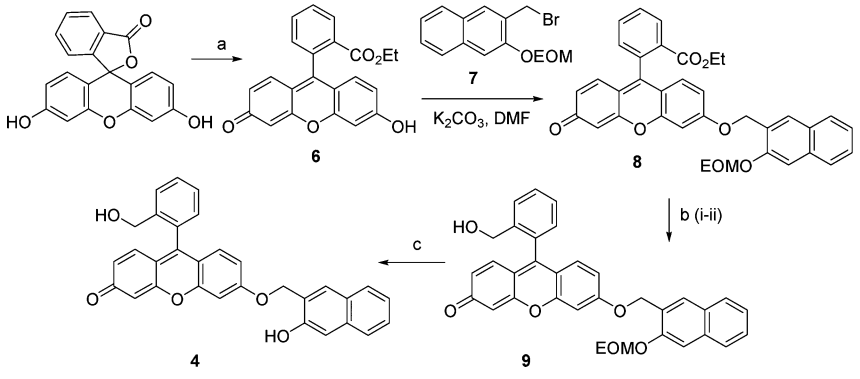


Figure 2. Emission spectra (λ_{exc} = 460 nm) of a 3.4 μM solution of **4** (in PBS buffer + 2% DMSO, pH 7.4) in the dark (dash-dotted line), after 10 min of irradiation at 300 nm (solid line), and after 20 min of exposure to 350 nm light (dashed line).

(Figure S1 in the Supporting Information).[23] Photochemical cleavage of the NQMP ether in caged dye **4** also resulted in a

Scheme 3. Synthesis of NQMP-Caged 6-Hydroxy-9-(2-(hydroxymethyl)phenyl)-3*H*-xanthen-3-one (4)[a]



[a]Reagents and conditions: (a) EtOH, $BF_3{\cdot}Et_2O$, reflux, 85%. (b) (i) DIBALH, CH_2Cl_2; (ii) DDQ, Et_2O; 52% over two steps. (c) Amberlyst-15, 95% methanol, 40 °C, 60%.

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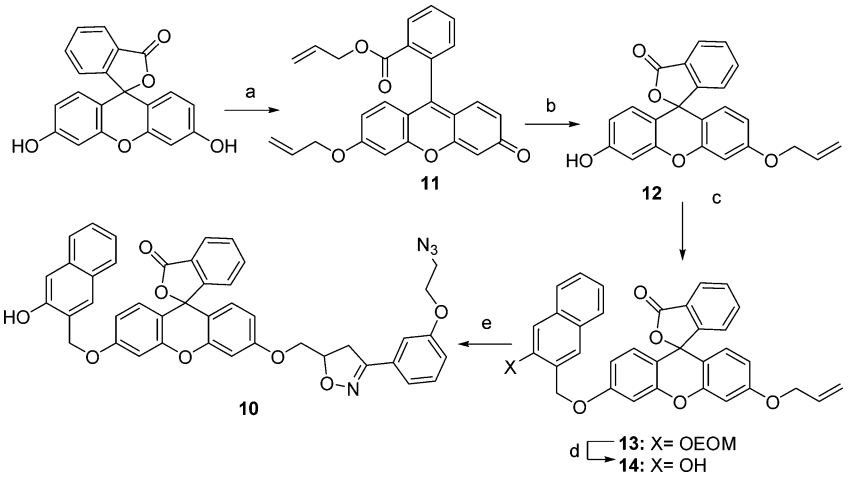
Scheme 4. Synthesis of Caged Fluorescein Derivative 10[a]



[a]Reagents and conditions: (a) Allyl bromide, $CsCO_3$, 60 °C, 94%. (b) NaOH (1 N), 95%. (c) **7**, Ag_2O, benzene, 77%. (d) Amberlyst-15, aq. MeOH, 50 °C, 74%. (e) **19**, Et_3N, CH_2Cl_2, 53%.

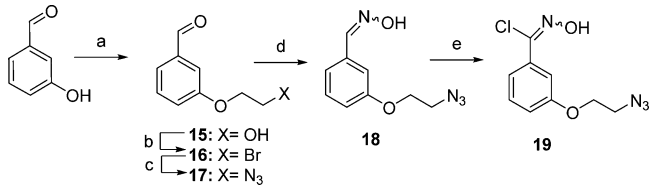
3−4-fold enhancement of the fluorescence efficiency (Φ_{fl} = 0.74−0.93; Figure 2). It is interesting to note that prolonged irradiation at 350 nm does not significantly affect the UV spectrum of **5** but results in the splitting of the emission band at 513 nm into two peaks (λ_{max} = 509 and 525 nm; Figure 2). This observation suggests that secondary photochemistry does not affect the fluorescent core of **5** but rather occurs in the aromatic side chain. We believe that this phenomenon is probably due to oxidation of the benzylic hydroxy group.

While photoactivation of caged dye **4** produces a dramatic enhancement of the fluorescence intensity, the maximum achievable contrast using this PAF is below 1:4 (dark vs exposed) due to significant emission of the PAF **4**. Complete quenching of the emission of fluorescein-based dyes can be achieved by etherification of both phenolic groups. Although the symmetrical caging of these moieties with photolabile protecting groups is a synthetically attractive approach, the removal of two protecting groups requires prolonged irradiation and increases the probability of photobleaching of the fluorophore. Incomplete uncaging, on the other hand, produces a mixture of three forms of the PAF, reducing the contrast and hampering quantitative measurements. Alternatively, only one phenolic hydroxyl can be caged with a photolabile protecting group, while the other is irreversibly capped. This strategy results in improved activation efficiency and excellent image contrast.[20b,24] Following this design, we have developed NQMP-caged fluorescein **10** bearing a "clickable" linker attached to the second phenyl ring. It is important to note that the order of alkylation of the phenolic hydroxy groups is crucial to the success of the synthesis of unsymmetrical fluorescein derivatives. Thus, alkylation of fluorescein with bromide **7** prevented the subsequent installation of the allyl group.

The working strategy for the preparation of PAF **10** is outlined in Scheme 4. Treatment of fluorescein with allyl bromide in the presence of cesium carbonate resulted not only in alkylation of one phenol moiety but also in lactone ring opening and the formation of allyl ester **11**. Subsequent saponification afforded the air-sensitive fluorescein lactone **12**. Alkylation of the second phenolic hydroxyl group in monoalkylated fluorescein is often complicated by the ring−chain tautomerism of the dye.[20b] However, silver(I) oxide-mediated alkylation of **12** with bromide **7** in benzene produced the target unsymmetrical bisalkylated fluorescein **13** in excellent yield. The removal of the 2-(ethoxymethoxy) protecting group was achieved in 74% yield using Amberlyst-15 resin in 95% aqueous methanol at 50 °C (Scheme 4).

The allyl group in NQMP-caged fluorescein **14** provides a convenient handle for subsequent functionalization or conversion into various reactive groups such as aldehydes, alcohols, and epoxides. We decided to exploit the dipolarophilic properties of this moiety and introduced an azide-terminated linker via 1,3-dipolar cycloaddition of a nitrile oxide. The latter was generated *in situ*[25] from *N*-hydroxycarboximidoyl chloride **19**. The nitrile oxide precursor **19** was synthesized from commercially available *m*-hydroxybenzaldehyde (Scheme 5).

Scheme 5. Preparation of 3-(2-Azidoethoxy)-*N*-hydroxybenzenecarboximidoyl Chloride (19)[a]



[a]Reagents and conditions: (a) (i) $TBSOCH_2CH_2Br$,[21] K_2CO_3, DMF, 46%; (ii) HF(aq), 87%. (b) Imidazole, Br_2, Ph_3P, CH_2Cl_2, 96%. (c) NaN_3, DMF, 69%. (d) $NH_2OH{\cdot}HCl$, CH_2Cl_2, 88%. (e) NCS, DMF, 65%.

Alkylation of the latter with TBDMS-protected bromoethanol followed by removal of the silyl protecting group afforded 3-(2-hydroxyethoxy)benzaldehyde (**15**). Bromination of aldehyde **15** followed by substitution with sodium azide produced azido aldehyde **17** in good yield. Treatment of aldehyde **17** with hydroxylamine hydrochloride gave the corresponding aldoxime **18** in 88% yield. The key intermediate 3-(2-azidoethoxy)-*N*-hydroxybenzenecarboximidoyl chloride **19** was obtained in 65% yield from aldoxime **18** using *N*-chlorosuccinimide.

Phosphate-buffered or aqueous methanol solutions of NQMP-caged fluorophore **10** have no detectable absorption bands above 350 nm (Figure 3). Irradiation of this solution with 300 nm fluorescent tubes (4 W) causes a rapid rise in the characteristic "quinoid" absorption bands at 452 and 496 nm due to release of fluorescein monoether **20** (Figure 3). Complete conversion is achieved in 5 min. Use of 350 nm lamps

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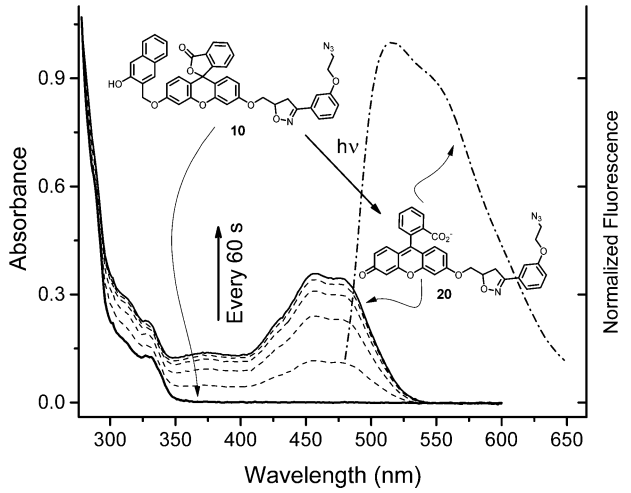

Figure 3. UV spectra of a 0.08 mM aqueous methanol solution of **10** in the dark (thick line) and during irradiation at 300 nm (dotted lines, every 60 s). Also shown is the emission spectrum (dash-dotted line, λ_{exc} = 460 nm) of a 2.3 μM solution of **20** (in PBS buffer + 9% DMSO, pH 7.4).

requires four times longer exposure, apparently because of the smaller extinction coefficient of the NQMP chromophore at this wavelength.[17] Upon excitation at 460 nm, the photolysate is intensely fluorescent (λ_{max} = 515 nm, Φ_{fl} = 0.22–25; Figure 3). The absorption spectrum shows two maxima above 400 nm, which also suggests the presence of tautomeric forms of uncaged dye **20**, typical of fluorescein derivatives.

To test the suitability of the azide moiety in **10** for the derivatization of various substrates using click chemistry, we evaluated its reactivity in the strain-promoted copper-free alkyne–azide cycloaddition (SPAAC) reaction. The rate of reaction of **10** with oxodibenzocyclooctyne (ODIBO, **21**)[26] was measured by UV spectroscopy at 25 ± 0.1 °C in methanol solution by following the decay of the characteristic 305 nm absorption of ODIBO (Figure 4). The observed rate constant of the reaction

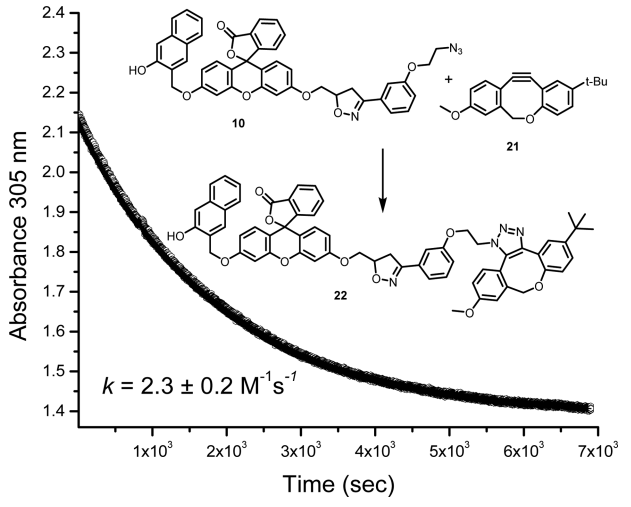

Figure 4. Changes in absorbance at 305 nm accompanying the reaction of ODIBO (**21**, 50 μM) with an azide moiety of NQMP-caged fluorophore **10** (0.22 mm) in methanol at 25.0 ± 0.1 °C.

between ODIBO (50 μM) and caged fluorophore **10** (0.22 mM) was k_{obs} = (5.11 ± 0.01) × 10^{-4} s^{-1}, which allowed us to estimate the bimolecular rate constant of this SPAAC reaction as k = 2.3 ± 0.2 M^{-1} s^{-1}. This value is similar to those observed in cycloadditions of aliphatic azides to ODIBO.[26] The formation of cycloadduct **22** was confirmed by HRMS of the product.[21]

In conclusion, we have demonstrated that the (3-hydroxy-2-naphthalenyl)methyl (NQMP) photolabile protecting group is suitable for the development of photoactivatable fluorophores. Two NQMP-caged derivatives of fluorescein were prepared following a relatively straightforward synthetic sequence. These caged compounds are nonfluorescent or have low emission in the dark. The irradiation of aqueous or methanol solutions of NQMP-caged fluorophores with low-intensity 300 and 350 nm light results in the efficient release of the fluorescent dye and a dramatic enhancement of the emission. The NQMP-caged fluorophore can be equipped with a reactive moiety for convenient attachment to a biological molecule, polymer, surface, or other substrate. We have demonstrated the derivatization of NQMP-caged fluorescein with an azide-terminated linker. The latter was subsequently utilized for copper-free ligation (SPAAC) to oxadibenzocyclooctyne. Feasibility studies of the use of NQMP-caged fluorescein **20** for fate mapping in zebrafish embryos are underway in our laboratory.

EXPERIMENTAL SECTION

General Methods. All reagents were purchased from commercial sources and used as received, unless otherwise noted. All organic solvents were dried and freshly distilled before use. Flash chromatography was performed using 40–75 μm silica gel. All NMR spectra were recorded in $CDCl_3$ using 400 MHz instruments and HRMS data were analyzed using ion trap or Q-TOF mass spectrometers, unless otherwise noted.

Photochemical Activation of PAFs 4 and 10. The reaction mixtures were irradiated in a merry-go-round photochemical reactor equipped with eight fluorescent UV lamps (4 W, 300 or 350 nm).

Fluorescence Measurements. Fluorescence measurements were conducted in aqueous buffers containing 2–9% DMSO as a cosolvent at concentrations of 2.3–3.4 μM. Emission spectra were recorded using 460 nm excitation light. The excitation source and the detector slits were set to 1 and 5 nm, respectively. Fluorescence quantum yields were determined using fluorescein in 0.1 N NaOH (Φ_{fl} = 0.95)[27] as the standard reference.

Kinetics. Accurate rate measurements were conducted in methanol at 25.0 ± 0.1 °C under pseudo-first-order conditions using a UV–vis spectrometer. The reaction of ODIBO with excess azide was monitored by the decay of cyclooctyne absorption at 305 nm.

Syntheses. *Ethyl 2-(6-Hydroxy-3-oxo-3H-xanthen-9-yl)benzoate (6).*[19] BF_3·OEt_2 (9.0 mL, 72 mmol) was added dropwise to a suspension of fluorescein (3.3 g, 8.9 mmol) in EtOH (30 mL), and the reaction mixture was refluxed overnight with an addition funnel loaded with molecular sieves attached. The reaction mixture was cooled, quenched with water, and diluted with EtOAc (100 mL). The aqueous layer was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with water (2 × 50 mL) and brine (20 mL) then dried over Na_2SO_4. The solvents were removed in vacuo to afford crude ethyl 2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoate (**6**) (3.2 g, 8.9 mmol, 99%) as a dark-red solid that was used without further purification. ^{1}H NMR (DMSO-d_6): δ 8.17 (d, J = 7.6 Hz, 1H), 7.85 (t, J = 7.3 Hz, 1H), 7.76 (t, J = 7.5 Hz, 1H), 7.48 (d, J = 7.2 Hz, 1H), 6.78 (d, J = 9.8 Hz, 2H), 6.54 (m, 3H), 3.95 (q, J = 7.0 Hz, 2H), 0.85 (t, J = 7.0 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ 166.2, 164.9, 157.1, 152.1, 135.0, 134.1, 131.7, 131.0, 130.5, 115.7, 104.3, 53.3, 41.1, 40.9, 40.7, 40.5, 40.3, 40.0, 39.8.

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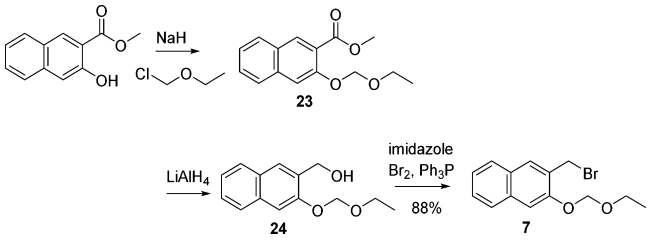
*Preparation of 2-Bromomethyl-3-(ethoxymethoxy)naphthalene (**7**).*



*(3-(Ethoxymethoxy)naphthalen-2-yl)methanol (**24**).* Sodium hydride (833 mg, 23 mmol) was added to an ice-cold solution of methyl 3-hydroxy-2-naphthoate (1.52 g, 7.5 mmol) in THF (80 mL). The mixture was stirred for 20 min, and chloromethyl ethyl ether (1.40 mL, 15 mmol) was added dropwise. The solution was allowed to warm to rt, stirred overnight, and quenched with 5% HCl (20 mL). The crude ether **23** was extracted with ether (3 × 50 mL). The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo.

Powdered lithium aluminum hydride (146 mg, 3.8 mmol) was added in portions to a solution of crude **23** (500 mg, 1.9 mmol) in 20 mL of THF at 0 °C. The mixture was allowed to warm to rt, stirred for 30 min, and quenched with EtOAc (40 mL) followed by HCl (5%, 10 mL). The aqueous layer was extracted with ether (3 × 50 mL) and the combined organic phases were washed with brine (20 mL). Solvents were removed in vacuo, and the crude product was purified by silica gel chromatography, eluting with 10−30% EtOAc in hexanes, to afford (3-(ethoxymethoxy)naphthalen-2-yl)methanol (**24**) (430 mg, 1.85 mmol, 96%) as a colorless oil. ^{1}H NMR: δ 7.76−7.74 (m, 3H), 7.45−7.35 (m, 3H), 5.37 (s, 2H), 4.85 (s, 2H), 3.76 (q, 2H), 2.78 (br s, 1H, OH),1.26 (t, 3H). ^{13}C NMR: δ 153.5, 134.1, 131.0, 129.3, 127.4, 126.3, 124.4, 109.0, 93.4, 64.8, 62.1, 15.3. HRMS-ESI calcd for $C_{14}H_{15}O_3^-$ $[M - H]^-$ 231.1027, found 231.0998.

*2-(Bromomethyl)-3-(ethoxymethoxy)naphthalene (**7**).* Br_2 (0.09 mL, 1.7 mmol) was added to an ice-cold solution of Ph_3P (440 mg, 1.7 mmol) and imidazole (114 mg, 1.7 mmol) in DCM (20 mL), and the mixture was stirred for 10 min. A solution of **24** (260 mg, 1.1 mmol) in DCM (40 mL) was added, and the resulting mixture was stirred for 30 min at 0 °C. The reaction mixture was filtered, and the precipitate was washed with cold 20% ether in hexane (2 × 5 mL). The combined filtrates were washed with a saturated solution of sodium dithionite (2 × 10 mL) and brine (20 mL), and the solvents were removed in vacuo. The crude residue was purified by silica gel chromatography, eluting with 10% ether in hexanes, to afford **7** (320 mg, 1.08 mmol, 97%) as a white solid. Mp = 54 °C. ^{1}H NMR: δ 7.83 (s, 1H), 7.44 (dd, J = 3.2, 8.0 Hz, 2H), 7.44 (m, 2H), 7.36 (dt, J = 1.2, 5.6 Hz, 1H), 5.45 (s, 2H), 4.72 (s, 2H), 3.84 (q, 2H), 1.28 (t, 3H). ^{13}C NMR: δ 153.1, 134.9, 130.5, 129.1, 128.1, 127.8, 127.1, 127.1, 124.6, 109.5, 93.3, 64.9, 29.7, 15.4. HRMS-ESI calcd for $C_{14}H_{16}BrO_2^+$ $[M + H]^+$ 295.0328, found 295.0342.

*Ethyl 2-[6-(3-(Ethoxymethoxy)naphthalen-2-yl)methoxy-3-oxo-3H-xanthen-9-yl]benzoate (**8**).* Potassium carbonate (0.051 g, 0.37 mmol) was added to a solution of fluorescein ethyl ester **6** (120 mg, 0.33 mmol) and bromide **7** (154 mg, 0.52 mmol) in DMF (5 mL) at rt. The mixture was stirred for 4 h, quenched with HCl (5%, 5 mL), and diluted with EtOAc (20 mL). The reaction mixture was washed with saturated NH_4Cl (3 × 20 mL) and brine (20 mL). The organic layer was dried with Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography, eluting with 1% MeOH in $CHCl_3$, to afford ethyl 2-[6-(3-(ethoxymethoxy)naphthalen-2-yl)methoxy-3-oxo-3*H*-xanthen-9-yl]benzoate (**8**) (0.19 g, 0.33 mmol, 99%) as a bright-yellow solid. R_f = 0.1, mp = 124 °C. ^{1}H NMR: δ 8.25 (dd, J = 1.2, 1.6, 6.4 Hz, 1H), 7.89 (s, 1H), 7.78−7.65 (m, 4H), 7.49 (s, 1H), 7.45 (dt, J = 1.2, 5.6 Hz, 1H), 7.36 (dt, J = 1.2, 1.6, 5.6 Hz, 1H), 7.30 (dd, J = 1.6, 2.4 Hz, 1H), 7.10 (d, J = 2.4 Hz, 1H), 6.93−6.85 (m, 3H), 6.54 (dd, J = 2.0, 7.6 Hz, 1H), 6.44 (d, 1H), 5.43 (s, 1H), 5.37 (s, 1H), 5.30 (dq, J = 1.6, 5.6 Hz, 2H), 3.80 (q, J = 7.2 Hz, 2H), 1.27 (t, 2H, OEt), 0.95 (t, 3H). ^{13}C NMR: δ 185.9, 165.6, 163.5, 159.1, 154.4, 152.9, 150.3, 134.4, 132.7, 130.6, 129.2, 128.1, 127.1, 125.8, 124.6, 117.9, 115.3, 114.2, 109.2, 106.0, 101.5, 93.5, 66.6, 64.9, 61.6, 15.4, 13.8. HRMS-ESI calcd for $C_{36}H_{31}O_7^+$ $[M + H]^+$ 575.2064, found 575.2070.

*6-((3-(ethoxymethoxy)naphthalen-2-yl)methoxy)-9-(2-(hydroxymethyl)phenyl)-8a,10a-dihydro-3H-xanthen-3-one (**9**).* A solution of DIBAL-H (5.5 mL, 5.5 mmol) was added dropwise to a solution of fluorescein ethyl ester **8** (663 mg, 1.10 mmol) in DCM (15 mL) at −78 °C under argon. The resulting solution was stirred for 10 min and allowed to warm to rt. The reaction mixture was quenched with 2 mL of a saturated solution of NH_4Cl and then stirred for 10 min. A solution of DDQ (274 mg, 1.21 mmol) in ether (50 mL) was added, and the mixture was stirred for another 30 min. The reaction mixture was filtered through a pad of Celite, and the solvents were removed in vacuo. The crude mixture was purified by chromatography (EtOAc/hexanes 1:1) to afford alcohol **9** (320 mg, 0.57 mmol, 52%) as a yellow oil. ^{1}H NMR: δ 7.89 (s, 1H), 7.75 (t, J = 6.8 Hz, 3H), 7.49 (s, 1H), 7.46−7.31 (m, 4H), 6.95−6.71 (m, 5H), 6.59 (s, 1H), 6.50 (d, J = 7.9 Hz, 1H), 5.45 (d, J = 2.3 Hz, 2H), 5.25 (s, 2H), 4.85 (s, 1H), 3.88 (t, 2H), 3.59 (t, 2H), 3.38 (m, 3H), 2.09 (s, 3H). ^{13}C NMR: δ 176.5, 153.5, 152.9, 139.3, 134.3, 134.3, 130.8, 130.1, 129.4, 129.3, 128.5, 128.1, 128.0, 127.9, 127.8, 127.0, 127.0, 126.6, 126.5, 124.5, 124.5, 109.2, 101.7, 93.8, 93.7, 77.6, 77.2, 76.9, 71.9, 71.8, 68.4, 68.1, 66.2, 62.3, 59.2, 20.8. HRMS calcd for $C_{35}H_{31}O_7^+$ $[M + H]^+$ 563.2064, found 563.2071.

*9-(2-(Hydroxymethyl)phenyl)-6-((3-hydroxynaphthalen-2-yl)-methoxy)-3H-xanthen-3-one (**4**).* Amberlyst-15 resin (100 mg) was added to **9** (100 mg, 0.18 mmol) in 15 mL of aqueous methanol (95:5 v/v). The mixture was stirred at 50 °C and monitored by TLC. After 2 h, the reaction mixture was cooled, and the solids were removed by filtration. Evaporation of the solvents under reduced pressure provided **4** (50 mg, 0.11 mmol, 60%). ^{1}H NMR (DMSO-d_6): 10.16 (s, 1H), 9.83 (s, 1H), 7.88 (s, 1H), 7.78 (d, J = 8.4 Hz, 1H), 7.69 (d, J = 8.4 Hz, 1H), 7.47−7.34 (m, 4H), 7.30−7.18 (m, 3H), 6.89 (d, J = 2.4 Hz, 2H), 6.84 (d, J = 8.7 Hz, 1H), 6.76 (dd, J = 14.9, 8.4 Hz, 3H), 6.58 (d, J = 2.3 Hz, 1H), 6.51 (dd, J = 8.6, 2.5 Hz, 1H), 5.41 (s, 1H), 5.23 (s, 4H). ^{13}C NMR (DMSO-d_6): 159.0, 158.0, 153.3, 150.5, 150.4, 145.2, 138.4, 134.0, 129.7, 127.4, 125.6, 123.0, 122.8, 117.5, 108.7, 104.2, 101.6, 70.5, 65.4. HRMS-ESI calcd for $C_{31}H_{23}O_5^+$ $[M + H]^+$ 475.1540, found 475.1544.

*Bis(allyl)fluorescein **11**.* Allyl bromide (2.60 mL, 30.1 mmol) was added to a solution of fluorescein (2.5 g, 7.52 mmol) and cesium carbonate (6.13 g, 18.81 mmol) in DMF (75 mL). The mixture was stirred at 60 °C overnight and then poured into cold water (150 mL), and the allyl ether ester of fluorescein precipitated. The orange precipitate was filtered off, washed with cold water (3 × 50 mL), and dried to afford bis(allyl)fluorescein **11** (2.82 g, 6.84 mmol, 91%) as a yellow solid, which was recrystallized from CCl_4. Mp = 150−152 °C (lit.[19a] 153−155 °C). ^{1}H NMR: δ 8.86 (d, J = 7.6 Hz, 1H), 7.74 (t, J = 7.6, 6.8 Hz, 1H), 7.68 (t, J = 7.6, 6.8 Hz, 1H), 7.31 (d, J = 7.2 Hz, 1H), 6.95 (d, J = 2 Hz, 1H), 6.89 (m, 2H), 6.76 (dd, J = 8.8, 2.4 Hz, 1H), 6.54 (dd, J = 9.6, 1.6 Hz, 1H), 6.45 (d, J = 1.6 Hz, 1H), 6.11−6.01 (m, 1H), 5.64−5.54 (m, 1H), 5.46 (d, J = 17.2 Hz, 1H), 5.36 (d, J = 10.4 Hz, 1H), 5.12 (d, J = 4.4 Hz, 1H), 5.08 (s, 1H), 4.65 (d, J = 5.2 Hz, 2H), 4.52−4.42 (m, 2H). ^{13}C NMR: δ 185.9, 165.2, 163.2, 159.1, 154.4, 149.6, 134.6, 132.8, 132.1, 131.4, 131.2, 130.7, 130.2, 129.9, 129.1, 119.4, 118.9, 118.0, 115.2, 113.9, 106.0, 101.4, 69.7, 66.2.

*6′-Allyloxyfluorescein (**12**).* A solution of sodium hydroxide (25.5 mL, 25.5 mmol) was added to a solution of **11** (750 mg, 1.82 mmol) in MeOH (40 mL). The mixture was stirred at rt for 1 h, and MeOH was evaporated. The reaction mixture was neutralized with 25 mL of 1.0 M HCl and extracted with DCM (3 × 20 mL). The organic layer was concentrated in vacuo, and the crude product was purified by chromatography (40% EtOAc/hexanes) to give **12** (644 mg, 1.73 mmol, 95%) as a yellow solid. Mp = 189−190 °C (lit.[24] 206−207 °C). ^{1}H NMR (DMSO-d_6): δ 10.16 (s, 1H), 8.00 (d, J = 7.5 Hz, 1H), 7.79 (t, J = 7.4 Hz, 1H), 7.72 (t, J = 7.4 Hz, 1H), 7.28 (d, J = 7.6 Hz, 1H), 6.94 (d, J = 2.4 Hz, 1H), 6.75−6.67 (m, 2H), 6.64 (d, J = 8.8 Hz, 1H), 6.57 (s, 2H), 6.10−5.96 (m, 1H), 5.41 (dd, J = 17.3, 1.5 Hz, 1H), 5.28 (d, J = 10.6 Hz, 1H), 4.64 (d, J = 5.2 Hz, 2H). ^{13}C NMR (DMSO-d_6): δ 168.5, 163.8, 159.8, 159.47, 152.4, 151.7, 151.7, 135.6, 133.2, 130.1, 129.0, 128.9, 126.0, 124.6, 123.9, 117.7, 112.7, 112.3, 111.1, 109.4, 104.2, 102.2, 101.6, 82.6, 68.5, 40.2, 39.9, 39.7, 39.5, 39.3, 39.1, 38.9.

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*3′-(Allyloxy)-6′-((3-(ethoxymethoxy)naphthalen-2-yl)methoxy)-3H-spiro[isobenzofuran-1,9′-xanthen]-3-one (**13**).* Silver(I) oxide (317 mg, 1.37 mmol) was added to a solution of **12** (300 mg, 0.81 mmol) and **7** (380 mg, 1.29 mmol) in dry benzene (10 mL). THF (2 mL) was added, and the mixture was refluxed for 16 h, allowed to cool, and filtered through a thin layer of Celite. The Celite was rinsed with EtOAc (80 mL). The solvents were removed in vacuo, and the residue was purified by chromatography (25% acetone/hexanes) to afford **13** (363 mg, 0.62 mmol, 77%) as a yellowish oil. ^{1}H NMR: δ 8.03 (d, J = 7.6 Hz, 1H), 7.90 (s, 1H), 7.78 (t, J = 7.6 Hz, 2H), 7.70−7.63 (m, 2H), 7.50−7.42 (m, 2H), 7.37 (t, J = 7.6 Hz, 1H), 7.19 (d, J = 7.5 Hz, 1H), 6.92 (d, J = 2.2 Hz, 1H), 6.79−6.68 (m, 4H), 6.66−6.61 (m, 1H), 6.11−5.98 (m, 1H), 5.45−5.41 (m, 3H), 5.34−5.26 (m, 3H), 4.57 (d, J = 5.3 Hz, 2H), 3.80 (q, J = 7.1 Hz, 2H), 1.30 (t, 3H). ^{13}C NMR: δ 169.6, 160.9, 160.5, 153.4, 153.0, 152.8, 152.7, 135.1, 134.4, 132.9, 129.9, 129.3, 129.2, 128.0, 128.0, 127.1, 127.0, 126.7, 126.9, 125.2, 124.5, 124.2, 118.3, 112.6, 112.4, 111.6, 111.7, 109.2, 102.1, 102.0, 93.5, 83.4, 77.6, 77.2, 76.9, 69.3, 66.3, 64.9, 15.4. HRMS-ESI calcd for $C_{37}H_{31}O_7^+$ [M + H]$^+$ 587.2064, found 587.2068.

*3′-(Allyloxy)-6′-((3-hydroxynaphthalen-2-yl)methoxy)-3H-spiro-[isobenzofuran-1,9′-xanthen]-3-one (**14**).* Amberlyst-15 resin (300 mg) and water (1 mL) were added to a solution of **13** (300 mg, 0.51 mmol) in MeOH (20 mL), and the mixture was stirred overnight at 50 °C. The Amberlyst was filtered off, and the solvents were removed in vacuo to afford crude **14** (201 mg, 0.38 mmol, 74%) as a pale-yellow solid, which was used without further purification. Mp = 92−95 °C. ^{1}H NMR (acetone-d_6): δ 9.11 (s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 7.95 (s, 1H), 7.82−7.77 (m, 2H), 7.75−7.66 (m, 2H), 7.45−7.35 (m, 2H), 1.15−1.09 (m, 1H), 7.31−7.25 (m, 3H), 6.86 (dd, J = 8.1, 1.8 Hz, 2H), 2.99−2.95 (m, 1H), 7.00 (d, J = 2.5 Hz, 1H), 6.77 (d, J = 8.8 Hz, 1H), 6.73 (d, J = 2.0 Hz, 2H), 6.13−6.03 (m, 1H), 5.43 (dd, J = 17.3, 1.7 Hz, 1H), 5.37 (s, 2H), 5.27 (dd, J = 10.6, 1.4 Hz, 2H), 4.67−4.61 (m, 2H). ^{13}C NMR (acetone-d_6): δ 169.5, 161.8, 161.4, 154.2, 154.0, 153.4, 153.3, 136.2, 135.6, 134.2, 130.9, 130.1, 130.0, 129.4, 129.2, 128.7, 127.8, 127.5, 127.2, 126.8, 125.5, 125.0, 124.2, 118.0, 113.4, 113.3, 112.8, 112.7, 110.1, 102.7, 102.7, 102.6, 102.5, 83.4, 69.8, 66.8. HRMS-ESI calcd for $C_{34}H_{25}O_6^+$ [M + H]$^+$ 529.1646, found 529.1656.

*3-(2-Hydroxyethoxy)benzaldehyde (**15**).*[28] K_2CO_3 (4.53 g, 33 mmol) was added to a solution of 3-hydroxybenzaldehyde (2.0 g, 16.4 mmol) in DMF (20 mL) at rt, and the mixture was stirred for 20 min. TBDMS-protected bromoethanol (4.70 g, 19.7 mmol) was added, and the mixture was stirred overnight. The reaction mixture was quenched with HCl (5%, 5 mL) and diluted with EtOAc (20 mL). The resulting mixture was washed with saturated NH_4Cl (3 × 20 mL) and brine (10 mL). The organic layer was then dried with Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified through a short plug of silica gel to afford 3-(2-((*tert*-butyldimethylsilyl)oxy)-ethoxy)benzaldehyde (4.20 g, 46%, considering that the product contained about 50% DMF by NMR) as a colorless oil. ^{1}H NMR 9.95 (s, 1H), 7.45−7.36 (m, 3H), 7.18 (dt, J = 6.8, 2.5 Hz, 1H), 3.87 (t, J = 6.5 Hz, 2H), 3.38 (t, J = 6.5 Hz, 2H), 0.89 (d, 15H).

Aqueous HF (0.47 mL, 14 mmol) was added to a solution of 3-(2-((*tert*-butyldimethylsilyl)oxy)ethoxy)benzaldehyde (2.0 g, 7.1 mmol) in acetonitrile (40 mL) at room temperature. The reaction mixture was stirred, and the progress of the reaction was monitored by TLC. After 15 min, solid $NaHCO_3$ (1.5 g, 14.3 mmol) was added. The solid $NaHCO_3$ was filtered and washed with ether (3 × 5 mL). The solvents were evaporated from the combined organic phases, and the residue was purified by flash chromatography (40% EtOAc/hexanes) to afford **15** (1.04 g, 6.2 mmol, 87%) as a colorless oil. ^{1}H NMR: δ 9.93 (s, 1H), 7.46−7.41 (m, 2H), 7.38−7.36 (m, 1H), 7.18 (dt, J = 7.3, 2.3 Hz, 1H), 4.12 (t, 2H), 3.98 (br s, 2H), 2.65 (br s, 1H). ^{13}C NMR: δ 192.2, 159.4, 137.9, 130.3, 124.0, 122.0, 113.1, 77.6, 77.2, 76.9, 69.7, 61.4.

*3-(2-Bromoethoxy)benzaldehyde (**16**).*[29] Br_2 (0.316 mL, 6.14 mmol) was added to an ice-cold solution of Ph_3P (1.503 g, 5.73 mmol) and imidazole (418 mg, 6.14 mmol) in DCM (10 mL), and the mixture was stirred for 10 min. A solution of **15** (680 mg, 4.1 mmol) in DCM (10 mL) was added dropwise, and the reaction mixture was stirred at 0 °C for 1 h. The precipitate was separated and washed with ether (2 × 50 mL). The combined organic layers were washed with a saturated solution of sodium dithionite (2 × 30 mL) and brine (20 mL) then dried over Na_2SO_4. The solvent was removed in vacuo, and the crude residue was purified by chromatography (20% EtOAc in hexanes) to afford **16** (900 mg, 3.9 mmol, 96%) as a colorless oil. ^{1}H NMR: δ 9.97 (s, 1H), 7.50−7.43 (m, 2H), 7.40−7.35 (m, 2H), 7.22−7.18 (m, 1H), 4.35 (t, J = 6.1 Hz, 2H), 3.66 (t, J = 6.1 Hz, 2H). ^{13}C NMR: δ 192.0, 162.88, 158.9, 138.1, 130.4, 124.3, 122.2, 113.2, 77.6, 77.2, 76.9, 68.3, 60.6, 53.6, 34.8, 31.7, 29.0, 22.8, 14.3.

*3-(2-Azidoethoxy)benzaldehyde (**17**).* Sodium azide (461 mg, 7.1 mmol) was added to a solution of **16** (650 mg, 2.8 mmol) in DMF (10 mL), and the mixture was stirred at 100 °C overnight. The reaction mixture was allowed to cool to rt, poured into a saturated solution of ammonium chloride (50 mL), and extracted with DCM (3 × 50 mL). The organic phase was washed with water (3 × 30 mL) then brine (20 mL) and dried over Na_2SO_4, and the solvent was removed in vacuo. The crude residue was passed through a short plug of silica gel (hexanes) to afford **17** (370 mg, 2.0 mmol, 71%) as a colorless oil. ^{1}H NMR: δ 9.97 (s, 1H), 7.52−7.43 (m, 2H), 7.42−7.37 (m, 2H), 7.24−7.19 (m, 1H), 4.23−4.19 (m, 2H), 3.63 (t, J = 4.9 Hz, 2H). ^{13}C NMR: δ 192.0, 159.0, 138.1, 130.4, 124.3, 122.2, 112.9, 77.6, 77.2, 76.9, 67.4, 50.2. HRMS-ESI calcd for $C_9H_{10}N_3O_2^+$ [M + H]$^+$ 192.0768, found 192.0760.

*3-(2-Azidoethoxy)benzaldehyde Oxime (**18**).* Hydroxylamine hydrochloride (191 mg, 2.8 mmol) was added to a solution of **17** (350 mg, 1.8 mmol) and Et_3N (0.383 mL, 2.8 mmol) in DCM (10 mL). The mixture was stirred at rt for 2 h and then diluted with water (10 mL). The aqueous layer was then extracted with DCM (3 × 15 mL), and the combined organic layers were washed with saturated $NaHCO_3$ (10 mL) and brine (5 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated. Purification by chromatography (20% EtOAc in hexanes) gave **18** (334 mg, 1.6 mmol, 88%) as a colorless oil. ^{1}H NMR: δ 8.71−8.68 (s, 1H), 8.17−8.11 (s, 1H), 7.36−7.30 (t, J = 7.8 Hz, 1H), 7.20−7.15 (m, 3H), 7.01−6.94 (dd, J = 8.2, 1.6 Hz, 1H), 4.20−4.15 (t, J = 5.0 Hz, 2H), 3.63−3.58 (t, J = 4.9 Hz, 2H). ^{13}C NMR: δ 158.7, 150.4, 133.6, 130.2, 121.0, 117.2, 112.0, 77.6, 77.2, 76.9, 67.2, 50.3. HRMS-ESI calcd for $C_9H_{11}N_4O_2^+$ [M + H]$^+$ 207.0877, found 207.0874.

*3-(2-Azidoethoxy)-N-hydroxybenzencarboximidoyl Chloride (**19**).* NCS (194 mg, 1.5 mmol) was added in portions to a solution of oxime **18** (300 mg, 1.5 mmol) in DMF (2 mL). About 1 mL of dilute gaseous HCl (from the headspace above conc. HCl) was bubbled into the solution to initiate the reaction. After 4 min, the temperature rose to 31 °C. In 20 min the temperature dropped to 25 °C, and the reaction mixture then was diluted with ether (20 mL). The reaction mixture was quenched with water (5 mL). Saturated NH_4Cl (10 mL) was added, and the reaction mixture was extracted with ether (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, and the solvent was removed under reduced pressure to give crude chloride **19** (227 mg, 0.94 mmol, 65%) as a pale-yellow oil. ^{1}H NMR: δ 10.39−10.37 (s, 1H), 7.50−7.47 (m, 1H), 7.40 (s, 1H), 7.34−7.27 (m, 1H), 7.02−6.95 (m, 1H), 4.20−4.15 (t, J = 5.0 Hz, 2H), 3.64−3.57 (t, J = 5.0 Hz, 2H). ^{13}C NMR: δ 158.3, 138.5, 134.5, 129.7, 120.6, 117.2, 113.1, 67.7, 67.3, 50.3, 50.2. HRMS-ESI calcd for $C_9H_{10}ClN_4O_2^+$ [M + H]$^+$ 241.0487, found 241.0487.

*NQMP-Caged Fluorescein Azide **10**.* A solution of TEA (0.04 mL, 0.25 mmol) in DCM (2 mL) was added dropwise to a stirred solution of **14** (90 mg, 0.17 mmol) and **19** (45 mg, 0.19 mmol) in DCM (3.5 mL), and the mixture was stirred overnight. The solvent was removed in vacuo, and the residue was purified by chromatography (DCM/EtOAc/hexanes 2:3:7) to afford **10** (66 mg, 0.090 mmol, 53%) as a pale-yellow fluffy solid. Mp = 92−93 °C. ^{1}H NMR (acetone-d_6): δ 9.09 (s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 7.95 (s, 1H), 7.82−7.67 (m, 4H), 7.48−7.27 (m, 7H), 7.07−7.05 (m, 1H), 7.00 (s, 1H), 6.91 (s, 1H), 6.88−6.86 (m, 1H), 6.85−6.74 (m, 3H), 5.37 (s, 2H), 5.20−5.08 (m, 1H), 4.31−4.23 (d, J = 4.1 Hz, 4H), 3.71−3.65 (t, J = 4.8 Hz, 2H), 3.64−3.36 (m, 2H). ^{13}C NMR: δ 168.7, 161.0, 160.8, 158.9, 156.4, 153.4, 153.2, 152.6, 134.9, 131.6, 130.1, 129.5, 129.5, 129.2, 129.2, 128.6, 127.0, 126.5, 126.5, 126.0, 112.5, 112.3, 112.0, 98.3, 82.5, 67.4, 66.0, 50.2. HRMS-ESI calcd for $C_{43}H_{33}N_4O_8^+$ [M + H]$^+$ 733.2293, found 733.2292.

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■ ASSOCIATED CONTENT

Supporting Information

^{1}H NMR, ^{13}C NMR, and HRMS spectra of newly synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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